REMOVAL OF MOLECULAR ASSAY INTERFERENCES

RELATED APPLICATIONS

This application is a continuation-in-part of copending Application No. 09/805,785, filed March 13, 2001, which is a continuation of Application No. 09/185,402, filed November 3, 1998, which is a continuation-in-part of Application No. 08/988,029, filed December 10, 1997. The entire contents of all the aforementioned applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present disclosure relates generally to the field of DNA analysis. More particularly, the present disclosure relates to methods and systems for removing interferences from test samples, e.g., DNA-containing samples obtained from living subjects, when they are submitted for or subjected to molecular assays.

The copying and cloning of virtually any nucleic acid sequence has been greatly facilitated by the polymerase chain reaction (PCR), which has become a fundamental methodology in molecular biology. In its simplest form, the PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences. In brief, the PCR involves hybridizing primers to the denatured strands of a target nucleic acid or template in the presence of a polymerase enzyme and nucleotides under appropriate reaction conditions. The polymerase enzyme (usually a thermostable DNA polymerase) then recognizes the primer hybridized to the template and processes a primer extension product complementary to the template. The resultant template and primer extension product can then be subjected to further rounds of subsequent denaturation, primer hybridization, and extension as many times as desired in order to increase (or amplify) the amount of nucleic acid which has the same sequence as the target nucleic acid. Commercial vendors market PCR reagents and publish PCR protocols. The PCR is capable of producing a selective enrichment of a specific DNA sequence by a factor of 10^9. The method is described in, e.g., U. S. Patent Nos. 4,683,202; 4,683,195; 4,800,159; and 4,965,188, and in Saiki et al., 1985, *Science* 230:1350.

The optimal efficiency of the amplification reaction, however, may be compromised by a number of unwanted side reactions. For example, many PCR procedures yield non-specific by-products caused by mispriming of the primers and template. Primers hybridizing to each other may also result in lost efficiency. This problem may be particularly acute when the target

nucleic acid is present in very low concentrations and may obscure any amplified target nucleic acid (*i.e.*, may produce high background).

Also, masking agents which interfere or inhibit such molecular assays as the PCR are a problem in the art. Such inhibitors, which include leukocyte esterases, heme proteins, e.g., myoglobin and hemoglobin analogs, oxidation and breakdown products such as ferritins, methemoglobin, sulfhemoglobin and bilirubin, affect the accuracy of the assay, masking the true or detectable amount of, e.g., DNA in the sample. It is also conceivable that, e.g., a human sample containing genetic material for analysis could be spiked or doped with such agents to render a molecular assay done on the sample less trustworthy, or inconclusive.

Modern testing and treatment procedures have successfully reduced the prevalence and severity of many infectious diseases. For example, sexually-transmitted disease (STD) clinics regularly screen and treat patients for such diseases as gonorrhea and syphilis. Infectious agents such as gonococci may be identified by analyzing a DNA sample. Genetic transformation tests (GTT), such as the Gonostat® procedure (Sierra Diagnostics, Inc., Sonora, CA), can be used to detect gonococcal DNA in specimens taken from the urethra of men, and the cervix and anus of women. *See, e.g.*, Jaffe et al., *Diagnosis of gonorrhea using a genetic transformation test on mailed clinical specimens*, J. Inf. Dis. **1982**; 146:275-279, and Whittington et al., *Evaluation of the genetic transformation test,*. Abstr. Ann. Meeting. Am. Soc. Microbiol. **1983**; p. 315. The Gonostat® assay is discussed in Zubrzycki et al., *Laboratory diagnosis of gonorrhea by a simple transformation test with a temperature-sensitive mutant of Neisseria gonorrhoeae,* Sex. Transm. Dis. **1980**; 7:183-187. The Gonostat(3) GTT, for example, may be used to detect, e.g., gonococcal DNA in urine specimens. The Gonostat assay uses a test strain, *N. Gonorrhoeae,* ATCC 31953, which is a mutant strain that is unable to grow into visible colonies on chocolate agar at 37°C in 5% CO_2. Gonococcal DNA extracted from clinical material can restore colony growth ability to this test strain.

Such tests such can be used to detect DNA in such bodily fluids and excretions as urine, blood, blood serum, amniotic fluid, spinal fluid, conjunctival fluid, salivary fluid, vaginal fluid, stool, seminal fluid, and sweat. Another test that can be used to identify DNA in a bodily fluid is the PCR, since it uses discrete nucleic acid sequences and therefore can be effective even in the absence of intact DNA.

SUMMARY OF THE INVENTION

The present invention relates to methods, systems and reagents for enabling and improving molecular assay of nucleic acids in bodily samples, e.g., fluids and excretions such as urine and blood to be carried out with greater sensitivity. It is believed, without limitation to a particular theory or view, that the methods and reagents remove or inactivate certain masking agents known to be interferents of molecular assays such as methemoglobin, such that they no longer interfere with the accuracy or sensitivity of the molecular assay. These methods and reagents have been found to also surprisingly increase the signal obtained with nucleic acid testing methods such as the polymerase chain reaction, LC_x, (Abbott Laboratories) and genetic transformation testing. An unexpected additional advantage of the invention is that hybridization in molecular assays such as nucleic acid testing methods is improved, compared to when such assays are carried out without employing the present invention.

In an embodiment, the invention relates to methods of suppressing the action of masking agents of molecular assays, with the result being that the assay may be carried out at a much higher confidence level. The masking agents that are present in a nucleic acid-containing test sample are suppressed by contacting the test sample with an amount of one or more divalent metal chelators like ethylenediaminetetraacetic acid or 1,2-*bis*(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, or salts thereof; and an amount of one or more chelator enhancing components like lithium chloride, guanidine, or sodium salicylate. The amounts of the divalent metal chelator(s) and the chelator enhancing component(s) are selected such that the masking agents are suppressed, and upon contact with the divalent metal chelator(s)/chelator enhancing component(s), the masking agents are suppressed. The amount of the divalent metal chelator is generally in the range of from about 0.001M to 0.1M, and the amount of the chelator enhancing component is generally in the range of from about 0.1M to 2M. The amount of chelator enhancing component is more desirably at least 1M, and the divalent metal chelator is desirably present in an amount of at least about 0.01M.

In another aspect, the invention relates to methods of improving the signal response of a molecular assay. The masking agents in a nucleic acid-containing test sample are suppressed by contacting the test sample with an amount of one or more divalent metal chelator(s); and an amount of one or more chelator enhancing components. The amounts of the divalent metal chelator(s) and chelator enhancing component(s) are selected such that the masking agents are

suppressed. Molecular analytes of interest from the preserved test sample are then extracted; and a molecular assay is conducted on the extracted molecular analytes of interest, whereupon the signal response of the molecular assay is improved. Signal response is believed to be enhanced in part due to enhanced hybridization as a result of the use of the reagents of the present invention.

A further aspect of the invention relates to methods of improving hybridization of nucleic acids, including contacting a test nucleic acid with a reagent comprising an amount of at least one divalent metal chelator, e.g., in the range of from about 0.001M to 0.1M; and an amount of at least one chelator enhancing component, e.g., lithium chloride, guanidine, sodium salicylate, sodium perchlorate, or sodium thiocyanate, e.g., in the range of from about 0.1M to 2M, such that a test solution is formed; and contacting the test solution with a target nucleic acid under conditions favorable for hybridization, such that hybridization occurs.

The methods and reagents of the invention may further include an amount of at least one enzyme-inactivating component such as manganese chloride, sarkosyl, or sodium dodecyl sulfate, generally in the range of about 0-5% molar concentration.

Accordingly, in one aspect, the invention provides a method for amplifying target nucleic acids, including combining a target nucleic acid under conditions which allow for an amplification reaction to occur. The invention may also be useful in commercial applications including specialty chemicals and instrumentation for utilizing this technology, e.g., probe based diagnostics, microarray/DNA Chip methods, PCR (e.g., hot-start PCR) hybridization and amplification, SNP analysis, and DNA sequencing. Other applications include drug discovery and the study of drug response genes (pharmacogenomics), drug delivery and therapeutics.

An advantage of the invention is that no manipulation of the reaction mixture need be done following initial preparation. Thus, the invention may be used in existing automated PCR amplification systems and with *in situ* amplification methods where the addition of reagents after the initial denaturation step is inconvenient or impractical.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 is a graph of DNA concentration in urine according to the prior art;

Fig. 2 is a graph of eight day serial data on urine according to the prior art;

Fig. 3 is a graph of DNA concentration in serum according to the prior art;

Fig. 4 is a graph showing the interference of methemoglobin on PCR absorbance in a PCR amplification assay on hepatitis B sequences MD03/06 in untreated serum;

Fig. 5 is a graph showing the improvement in attenuating the interference of methemoglobin on PCR absorbance in a PCR amplification assay on hepatitis B sequences MD03/06 in serum which has been treated with a preservative of the invention; and

Fig 6 illustrates the synergistic effect provided by the components of the inventive reagents in protecting hepatitis B sequences in serum stored at room temperature and subsequently subjected to MD03/06 PCR detection;

Fig. 7 graphically illustrates a comparison of signal response in PCR assays wherein the DNA has been treated with a reagent of the invention, and one which has not;

Fig. 8 illustrates the efficacy of reagents of the present invention to enhance signal response of a branched DNA assay of blood plasma samples subjected to various storage conditions;

Fig. 9 illustrates the efficacy of reagents of the present invention to enhance signal response of a branched DNA assay of blood serum and plasma samples;

DETAILED DESCRIPTION OF THE INVENTION

"Molecular assay" includes nucleic acid amplification techniques such as the PCR; RT-PCR (e.g., U.S. Patent No. 4,683,202); LCR (ligase chain reaction) described in, e.g., EP-A-0320308; the "NASBA" or "3SR" technique described in, e.g., *Proc. Natl. Acad. Sci.* Vol. 87 pp. 1874-1878 March 1990 and *Nature* Vol. 350, No. 634. PP 91-92 Mar. 7, 1991; the "SDA" method described in, e.g., *Nucleic Acid Research*, Vol. 20 PP 1691-1696; $LC_{x,}$; and genetic transformation testing (GTT)

"Masking agents" or "interferents of molecular assay(s)" includes compounds which interfere or otherwise affect the accuracy of the assay, masking the true or detectable amount of the nucleic acid in the sample. Examples are leukocyte esterases, heme proteins such as myoglobin and hemoglobin analogs, derivatives, oxidation and breakdown products such as ferritins, methemoglobin, sulfhemoglobin and bilirubin.

"Metal cations" include cations associated with metal dependent enzymes. Examples of metal cations include cations of iron, aluminum, copper, cobalt, nickel, zinc, cadmium,

magnesium, and calcium. Metal cations of particular interest include magnesium (e.g., Mg^{+2}) and calcium (e.g., Ca^{+2}).

"Bodily fluid" includes e.g., urine, blood, blood serum, amniotic fluid; cerebrospinal and spinal fluid; fluid; synovial fluid; conjunctival fluid; salivary fluid; vaginal fluid; stool; seminal fluid; lymph; bile; tears, and sweat.

"Sample" includes substances containing or presumed to contain nucleic acid and may include a sample of tissue or fluid isolated from an individual or individuals, including bodily fluids, skin, blood cells, organs, tumors, and also to samples of *in vitro* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, recombinant cells and cell components).

"Divalent metal chelator" includes compounds which chelate or remove divalent metal cations such that metal dependent enzymes such as deoxyribonucleases are inactivated. Deoxyribonucleases, e.g., have been found to inactivate gonococcal DNA in urine over time. Suitable divalent metal chelators include ethylenediaminetetraacetic acid (EDTA), imidazole, ethylene*bis*(oxyethylenenitrilo)]tetraacetic acid (EGTA), [ethylene*bis*(oxyethylenenitrilo)]tetraacetic acid (EGTA); iminodiacetate (IDA); or 1,2-*bis*(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA); *bis*(5-amidino-2-benzimidazolyl)methane (BABIM) or salts thereof. Preferred divalent metal chelators include EDTA and BAPTA. The amount of the divalent metal chelator is generally present in a reagent solution the range of from about 0.001M to 0.1M. More desirably, the amount of the divalent metal chelator in the reagent solution is at least 0.01M.

"Chelator enhancing component" includes compounds which, *inter alia,* assist the divalent metal chelator in protecting the nucleic acids in the fluid. These chelator enhancing components are believed to inactivate metal independent enzymes found in samples, such as DNA ligases, e.g., D4 DNA ligase; DNA polymerases such as T7 DNA polymerase; exonucleases such as exonuclease 2, -exonuclease; kinases such as T4 polynucleotide kinase; phosphatases such as BAP and CIP phosphatase; nucleases such as BL31 nuclease and XO nuclease; and RNA-modifying enzymes such as *E coli* RNA polymerase, SP6, T7, T3 RNA polymerase, and T4 RNA ligase. Lithium chloride, guanidine, sodium salicylate, sodium perchlorate, and sodium thiocyanate have been found to be particularly effective. The amount of the chelator enhancing component is generally in the range of from about 0.1M to 2M, and

more desirably the amount of chelator enhancing component in the reagent solution is at least 1M.

"Nucleic acid", "polynucleotide" and "oligonucleotide" include DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), analogs of the DNA or RNA generated using nucleotide analogs or using nucleic acid chemistry, and PNA (protein nucleic acids); modified nucleotides such as methylated or biotinylated nucleotides, primers, probes, oligomer fragments, oligomer controls and unlabeled blocking oligomers polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), and any other type of polynucleotide which is an N-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. There is no intended distinction in length between the term "nucleic acid", "polynucleotide" and "oligonucleotide", and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA. Oligonucleotides typically include a sequence of approximately at least 6 nucleotides, preferably at least about 10-12 nucleotides, and more preferably at least about 15-20 nucleotides corresponding to a region of the designated nucleotide sequence.

Oligonucleotides are not necessarily physically derived from any existing or natural sequence but may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription or a combination thereof. Oligonucleotides or nucleic acids can include those which, by virtue of its origin or manipulation: (1) are not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) are linked to a polynucleotide other than that to which it is linked in nature; and (3) are not found in nature.

"Corresponding" means identical to or complementary to the designated sequence.

"Primer" or "nucleic acid primer" may refer to more than one primer and includes oligonucleotides, whether occurring naturally, as in a purified restriction digest, or produced synthetically, which are capable of acting as a point of initiation of synthesis along a complementary strand when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is catalyzed. Primers are typically between about 10 to 100 bases and are designed to hybridize with a corresponding template nucleic acid. Primer molecules may be complementary to either the sense or the anti-sense strand of a template nucleic acid and are typically used as complementary pairs that flank a

nucleic acid region of interest. Synthesis conditions include the presence of four different deoxyribonucleoside triphosphates and a polymerization-inducing agent such as DNA polymerase or reverse transcriptase, in a suitable buffer ("buffer" includes substituents which are cofactors, or which affect pH, ionic strength, etc.), and at a suitable temperature. The primer is preferably single-stranded for maximum efficiency in amplification.

The "complement" of a nucleic acid sequence includes oligonucleotides which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Certain bases not commonly found in natural nucleic acids may be included, for example, inosine and 7-deazaguanine. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, base composition and sequence of the oligonucleotide, ionic strength, and incidence of mismatched base pairs.

"Target sequence" or "target nucleic acid sequence" refers to a region of the oligonucleotide which is to be either amplified, detected or both. The target sequence resides between the two primer sequences used for amplification.

"Probe" refers to a labeled oligonucleotide which forms a duplex structure with a sequence in the target nucleic acid, due to complementarity of at least one sequence in the probe with a sequence in the target region. The probe, preferably, does not contain a sequence complementary to sequence(s) used to prime the polymerase chain reaction. Generally the 3' terminus of the probe will be "blocked" to prohibit incorporation of the probe into a primer extension product. "Blocking" can be achieved by using non-complementary bases or by adding a chemical moiety such as biotin or a phosphate group to the 3' hydroxyl of the last nucleotide, which may, depending upon the selected moiety, serve a dual purpose by also acting as a label for subsequent detection or capture of the nucleic acid attached to the label. Blocking can also be achieved by removing the 3'-OH or by using a nucleotide that lacks a 3'-OH such as a dideoxynucleotide.

"Polymerase" includes any one of, or a mixture of, the nucleotide polymerizing enzymes *E. coli* DNA polymerase I, TAQ polymerase, Klenow fragment of *E. coli* DNA

polymerase I, T4 DNA polymerase, reverse transcriptase where the template is RNA and the extension product is DNA, or a thermostable DNA polymerase.

"Thermostable nucleic acid polymerase" refers to an enzyme which is relatively stable to heat when compared, for example, to nucleotide polymerases from *E. coli* and which catalyzes the polymerization of nucleoside triphosphates. Generally, the enzyme will initiate synthesis at the 3'-end of the primer annealed to the target sequence, and will proceed in the 5'-direction along the template, and if possessing a 5' to 3' nuclease activity, hydrolyzing intervening, annealed probe to release both labeled and unlabeled probe fragments, until synthesis terminates. A preferred thermostable enzyme isolated from *Thermus aquaticus* (Taq) is described in U. S. Patent No. 4,889,818 and a method for using it in conventional PCR is described in, e.g., Saiki et al., 1988, *Science* 239:487. Taq DNA polymerase has a DNA synthesis-dependent, strand replacement 5'-3' exonuclease activity (see Gelfand, "Taq DNA Polymerase" in *PCR Technology: Principles and Applications for DNA Amplification*, Erlich, Ed., Stockton Press, N.Y. (1989), Chapter 2). Additional representative temperature stable polymerases include polymerases extracted from the thermostable bacteria *Thermus flavus, Thermus ruber, Thermus thermophilus, Bacillus stearothermophilus, Thermus lacteus, Thermus rubens, Thermotoga maritima, Thermococcus litoralis, Methanothermnus fervidus, Thermus filiformis, Thermus flavus, Pyrococcus furiosus, Thermococcus literolis*, a *Thermotoga* species, or a recombinant form thereof.

"Thermal cycle" includes any change in the incubation temperature of a nucleic acid sample designed to change the activity of a component of the sample such as, *e.g.*, the binding affinity of a primer for a nucleic acid.

The terms "hybridize" or "hybridization" are art-recognized and include the hydrogen bonding of complementary DNA and/or RNA sequences to form a duplex molecule. Typically, hybridization takes place between a primer and template but may also take place between primers and these reactions, when undesired or unscheduled, can be inhibited by using methods and compositions of the invention.

The terms "amplification" or "amplify" include the reactions necessary to increase the number of copies of a nucleic acid sequence, such as a DNA sequence. For the purposes of the present disclosure, amplification refers to the *in vitro* exponential increase in copy number of a target nucleic acid sequence, such as that mediated by a polymerase amplification reaction such

as the PCR. Other amplification reactions encompassed by the invention include RT-PCR (see, *e.g.*, U. S. Patent No. 4,683,202; Mullis *et al.*), and the ligase chain reaction (Barany, *Proc. Natl. Acad. Sci. USA* 88:189-193 (1991)).

"Selective amplification" refers to the preferential copying of a target or template nucleic acid of interest using a polymerase amplification reaction, such as the PCR.

The practice of the invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology and recombinant DNA techniques, which are within the skill of those in the art. Such techniques are explained fully in the literature. *See, e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins, eds., 1984); *A Practical Guide to Molecular Cloning* (B. Perbal, 1984); and a series, *Methods in Enzymology* (Academic Press, Inc.).

The reagents of the invention have surprisingly been found to remove the interference of masking agents, e.g., heme proteins including methemoglobin on PCR assays run on blood serum. Figures 4 and 5 illustrate the improvement obtained by use of the reagents disclosed herein. Increasing amounts of methemoglobin were spiked into untreated fresh human serum, to a concentration of 10dl/ml. Serial PCR assays were run over a four hour period.

Figure 6 illustrates the surprising and synergistic effect obtained by the combination of divalent metal chelators and chelator enhancing components in the inventive reagent (i.e., 1M sodium perchlorate/0.01M EGTA) in protecting hepatitis B sequences in serum stored at room temperature and subsequently subjected to MD03/06 PCR detection. The protocol run was as above (i.e., as illustrated in Figure 6.) It can be seen from the figures that compared to the addition of EGTA or sodium perchlorate individually, protection of Hep B sequences is dramatically increased when reagent solutions of the present invention are used.

In an advantageous embodiment, the invention also enables the molecular assay of nucleic acids in other bodily fluids and excretions to be carried out with greater sensitivity, as the methods and reagents of the invention have been found to surprisingly increase the signal obtained with such molecular assays as the PCR. Additionally, hybridization in such nucleic acid testing methods is unexpectedly improved.

The methods and preservatives of the invention may further include an amount of at least one enzyme inactivating component such as manganese chloride, sarkosyl, or sodium dodecyl sulfate, generally in the range of about 0-5% molar concentration.

The reagents of the invention may be used advantageously to prokaryotic, e.g., gonococcal DNA, although the teachings of the invention may be readily applied to the preservation of other types of DNA, including human, bacterial, fungal, and viral DNA, as well as to RNA. The reagents of the invention are believed to function by *inter alia,* inactivating two classes of enzymes present in bodily fluids such as blood or urine which the inventor has recognized as destructive to DNA integrity, metal-dependent and metal independent enzymes.

The methods and reagents of the invention have been found to increase the signal obtained with such nucleic acid testing methods as the polymerase chain reaction (PCR), LC_x, and genetic transformation testing (GTT). The invention has been found to surprisingly and unexpectedly enhance hybridization in such nucleic acid testing methods as the PCR. Figures 7 and 8 illustrate the improvement in hybridization obtained by use of a reagent disclosed herein on the hybridization of penicillinase-producing *Neisseria gonorrhea* (PPNG) DNA and PPNG-C probe.

A further aspect of the invention relates to methods of improving hybridization of nucleic acids, including contacting a test nucleic acid with a nucleic acid reagent solution comprising an amount of a divalent metal chelator in the range of, e.g., about 0.001M to 0.1M; and an amount of at least one chelator enhancing component in the range of, e.g., about 0.1M to 2M, such that a test solution is formed; and contacting the test solution with a target nucleic acid under conditions favorable for hybridization, such that hybridization occurs.

Figures 8 and 9 illustrate the efficacy of the methods and reagents of the invention in improving the results obtained with nucleic acid testing methods, in this case, a branched DNA assay (Chiron). In the tests run in Fig. 8, the bDNA assay was used to assess the effect of the reagents of the invention. DNA sequences from the hepatitis C virus were spiked into serum and plasma. The treated serum and plasma were mixed with 9ml of serum or plasma and 1ml of reagent. The following formulations were used: 1) 1M guanidine HCl/0.01M EDTA, 2) 1M sodium perchlorate/0.01M BAPTA, 3) 1M sodium thiocyanate/0.01M EGTA, and 4) 1M lithium chloride/0.01M EGTA. The formulations were stored for seven days at 4°C. bDNA assay relies on hybridization; it can clearly be seen from the absorbance results that the more

than doubling of the absorbance results indicates an enhancement of hybridization/annealing of the target sequences.

Fig. 9 illustrates a serum v. plasma study. 50ml samples of fresh human plasma, and 1ml samples of fresh human serum were treated with 1M guanidine HCL/0.01M EDTA and the bDNA assay was run on these samples after the samples were stored at 20°F for 48 hours. Results were compared to untreated samples. It can clearly be seen from the absorbance results that the more than doubling of the absorbance results indicates an enhancement of hybridization/annealing of the target sequences.

The invention has the advantage of being conveniently incorporated into established protocols without the need for extensive re-optimization.

In a preferred method, the PCR process is carried out as an automated process utilizing a thermostable enzyme. The reaction mixture is cycled through a denaturing step, a probe and primer annealing step, and a synthesis step, whereby cleavage and displacement occurs simultaneously with primer-dependent template extension. A DNA thermal cycler, which is specifically designed for use with a thermostable enzyme, may be employed.

Detection or verification of the labeled oligonucleotide fragments may be accomplished by a variety of methods and may be dependent on the source of the label or labels employed. One convenient embodiment of the invention is to subject the reaction products, including the cleaved labeled fragments, to size analysis. Methods for determining the size of the labeled nucleic acid fragments are known in the art, and include, for example, gel electrophoresis, sedimentation in gradients, gel exclusion chromatography and homochromatography.

During or after amplification, separation of the labeled fragments from the PCR mixture can be accomplished by, for example, contacting the PCR mixture with a solid phase extractant (SPE). For example, materials having an ability to bind oligonucleotides on the basis of size, charge, or interaction with the oligonucleotide bases can be added to the PCR mixture, under conditions where labeled, uncleaved oligonucleotides are bound and short, labeled fragments are not. Such SPE materials include ion exchange resins or beads, such as the commercially available binding particles Nensorb (DuPont Chemical Co.), Nucleogen (The Nest Group), PEI, BakerBond™ PEI, Amicon PAE 1000, Selectacel™ PEI, Boronate SPE with a 3'-ribose probe, SPE containing sequences complementary to the 3'-end of the probe, and hydroxylapatite. In a

specific embodiment, if a dual labeled oligonucleotide comprising à 3' biotin label separated from a 5' label by a nuclease susceptible cleavage site is employed as the signal means, the PCR amplified mixture can be contacted with materials containing a specific binding partner such as avidin or streptavidin, or an antibody or monoclonal antibody to biotin. Such materials can include beads and particles coated with specific binding partners and can also include magnetic particles.

Following the step in which the PCR mixture has been contacted with an SPE, the SPE material can be removed by filtration, sedimentation, or magnetic attraction, leaving the labeled fragments free of uncleaved labeled oligonucleotides and available for detection.

For detecting the resultant PCR product, any art recognized technique may be used, such as agarose gel electrophoresis, as described herein. Alternatively, the resultant products of the amplification reaction may be detected using a detectable label, that is, *e.g.*, isotopic, fluorescent, colorimetric, or detectable e.g., using antibodies. Accordingly, the amplification methods of the invention may be advantageously used to amplify virtually any target nucleic acid such as a nucleic acid fragment, gene fragment (*e.g.*, an exon or intron fragment), cDNA, or chromosomal fragment.

Genotyping by SNP (single nucleotide polymorphism) analysis and allele-specific oligonucleotide (ASO) hybridizations, which are the basis for microarray or DNA-Chip methods, are other genomic methods that are expected to benefit from a technology for enhanced accuracy of hybridization. Microarrays are constructed by arraying and linking PCR amplified cDNA clones or genes to a derivatized glass plate. Currently, the linking chemistries depend on high-salt buffers with formamide or dimethyl sulfoxide (DMSO) to denature the DNA and provide more single-stranded targets for eventual hybridization with high specificity and minimal background. This is a critical step in the preparation of reproducible, high-fidelity microarrays which may benefit from reversibly modified nucleic acids developed in this project. Further, the specific conditions of the pre-hybridization and hybridization steps can dramatically affect the signal from the microarray and technology from this project may be able to improve microarray performance at this step of the process.

Diagnostic Applications

The methods, compositions, and kits of the invention are useful in a variety of diagnostic applications, such as the amplification and detection of nucleic acid sequences found

in genomic DNA, bacterial DNA, fungal DNA, or viral RNA or DNA. The invention may also be used to detect or characterize nucleic acid sequences associated with infectious diseases (e.g., gonorrhea, chlamydia), genetic disorders, or cellular disorders such as cancer; or for the detection of certain types of non-genetic diseases (e.g., to detect the presence of a viral nucleic acid molecule (*e.g.*, HIV or hepatitis) within a nucleic acid sample derived from a human cell sample). Surface analysis, e.g., through the use of microarrays or gene chips, to detect the possible presence of, e.g., biowarfare agents, can be aided through the practice of the present invention.

Forensic Applications

Forensic science is concerned with the scientific analysis of evidence from a crime. Forensic biology applies the experimental techniques of molecular biology, biochemistry, and genetics to the examination of biological evidence for the purpose, for example, of positively identifying the perpetrator of a crime. Typically, the sample size of such biological evidence (*e.g.* hair, skin, blood, saliva, or semen) is very small and often contains contaminants and interferents of molecular assays. Accordingly, the techniques of the invention may be advantageously used to detect, *e.g.*, the sex or species of origin of even minute biological samples.

Research Applications

The methods and compositions of the invention have a variety of research applications. For example, they are useful for any research application in which genetic analyses must be performed on limited amounts of nucleic acid sample.

In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, recombinant DNA technology, PCR technology, immunology, and any necessary cell culture or animal husbandry techniques, which are within the skill of the art and are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch and Maniatis, *Molecular Cloning: Cold Spring Harbor Laboratory Press* (1989); *DNA Cloning*, Vols. 1 and 2, (D.N. Glover, Ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait, Ed. 1984); *PCR Handbook Current Protocols in Nucleic Acid Chemistry*, Beaucage, Ed. John Wiley & Sons (1999) (Editor); *Oxford Handbook of Nucleic Acid Structure*, Neidle, Ed., Oxford Univ Press (1999); *PCR Protocols: A Guide to Methods and Applications*, Innis *et al.*, Academic Press (1990); *PCR Essential Techniques: Essential Techniques*, Burke, Ed., John

Wiley & Son Ltd (1996); *The PCR Technique: RT-PCR,* Siebert, Ed., Eaton Pub. Co. (1998); *Quantitative PCR Protocols*, Kochanowski *et al.*, Eds., Humana Press (1999); *Clinical Applications of PCR*, Lo, Ed., Humana Press (1998); *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, John Wiley & Sons (1992); *Large-Scale Mammalian Cell Culture Technology,* Lubiniecki, A., Ed., Marcel Dekker, Pub., (1990); and *Manipulating the Mouse Embryo*, Hogan *et al.*, C.S.H.L. Press, Pub (1994).

The following exemplification is included for purposes of illustration and should not be construed as limiting the invention.

EXAMPLE 1

PCR Detection of Penicillinase-producing Neisseria gonorrhea

The PCR signal-enhancing effect of the reagents of the invention is demonstrated by the following example. Four varieties of TEM-encoding plasmids are found in PPNG. These are the 6.7kb (4.4Mda) Asian type, the 5.1kb (3.2Mda) African type, the 4.9kb (3.05-Mda) Toronto type and the 4.8kb (2.9-Mda) Rio Type. This PCR assay for PPNG takes advantage of the fact that the TEM-1 gene is located close to the end of the transposon Tn2; by the use of one primer in the TEM-1 gene and the other in a sequence beyond the end of Tn2, and common to all four plasmids, a PCR product only from plasmids and not from TEM-1 encoding plasmids was obtained. (Table 1, below) The conditions associated with this protocol were modified to include the reagent of the invention in the hybridization and the treated probe was mixed with the 761-bp amplification product per standard PCR protocol. The results were read at A_{450nm}.

Materials and Reagents:

- BBL chocolate II agar plates
- Sterile Tris Buffer 10 mM Tris (pH 7.4), 1mM EDTA
- 0.5-ml Gene Amp reaction tubes
- Sterile disposable Pasteur pipette tips
- Aerosol-resistant tips
- PCR master mix:
  - 50mM KCL
  - 2mM MgCl
  - 50μM each of
    - Deoxyribonucleoside triphosphate;

- 2.5 U of Taq Polymerase (Perkin Elmer);
- 5% glycerol;
- 50pmol each of primers PPNG-L and PNG-R (per 100 µl reaction)

- Denaturation solution
  - 1M Na 5x Denhardt's solution

- Prehybridization Solution
  - 5xSSC(1xSSc is 0.015 M NaCl plus 0.015 M sodium citrate);
  - 5x Denhardt's solution;
  - 0.05% SDS;
  - 0.1% Sodium Ppi, and
  - 100 µg of sonicated salmon sperm DNA per ml.

- Hybridization Solution
  - Same as prehybridization solution but without Denhardt's solution and including 200 µl of a reagent of the invention.
- 1ml of a reagent of the invention (1M guanidine HCl/0.01M EDTA, "Reagent 1")
- Avidin-HRP peroxidase complex (Zymed)
- Magnetic microparticles (Seradyne)

Table 1

Function	Name	Nucleotide sequence 5' to 3'
Primer	PPNG-L	AGT TAT CTA CAC GAC GG
Primer	PPNG-R	GGC GTA CTA TTC ACT CT
Probe	PPNG-C	GCG TCA GAC CCC TAT CTA TAA ACT C

Methods:

Sample preparation: 2 colonies were picked from a chocolate agar plate. Colonies were suspended in DI water just prior to setting up PCR. The master mix was prepared according to the recipe above. 5µl of the freshly prepared bacterial suspension was added to 95µl of master mix. The DNA was liberated and denatured in a thermocycler using three cycles of 3 min at 94°C and 3 min at 55°. The DNA was amplified in the thermal cycler by using a two step profile: a 25s denaturation at 95°C and a 25s annealing at 55°C for a total of thirty cycles. The

time was set between the two temperature plateaus to enable the fastest possible annealing between the two temperatures. 15 pmol of labeled (avidin-HRP complex) detection probe PPNG-C was added to the hybridization solution bound to magnetic micro particles with and without the preservative reagent at 37°C for 1 hour. The control and treated probes were then added to the amplification product and the reaction was colorimetrically detected at A_{450nm}. The signal obtained from the hybridization probes treated with a reagent of the invention was found to be significantly higher than the untreated probes.

EXAMPLE 2

Inhibition of amplification is a significant problem with STD specimens from both cervical and urethral sites. Based on a review of the literature, estimates of inhibition range from 2-20% for specimens collected with a swab. This experiment compares a novel swab collection device containing a reagent of the invention to a standard dry swab collection device and demonstrates that reagents of the invention can be utilized to significantly minimize the effects of inhibition, thereby reducing the incidence of false negative results.

The swab device used was a sterile polyurethane sponge impregnated with 700μl of the reagent of Example 1, which is housed in the bottom of an empty sterile tube. The specimen is collected on a separate sterile rayon swab and inserted into the above tube (Starplex). Once the swab has been inserted in the tube, the swab comes into contact with the sponge and absorbs the reagent, which treats the specimen accordingly. The control device used for comparison was a standard dry rayon swab in a sterile tube (Copan Diagnostics #155C -160C)

Four known amplification assays were included in this study: LCx® (Abbott Diagnostics), Probe-Tec® (BD Diagnostic Systems), TMA™ (Gen-Probe), and PCR® (Roche Diagnostics). Four separate laboratories were utilized to conduct the experiment, one for each assay platform.

Specimens were collected at four separate STD clinics using best-practice collection methods. At each collection site, 50 patients provided duplicate specimens for an aggregate of 200 treated samples and 200 untreated samples. All samples were transported to the laboratory at room temperature and processed within 8 hours of collection.

Current assay reagents and direction inserts were used to perform the amplification assay. A second amplified assay was utilized to challenge all positives to confirm that they were really true positives. LCx was refereed by PCR, and SDA, TMA, and PCR were all

refereed by LCx. Additionally, all positive extracts that were untreated (dry) were subjected to GC/MS analysis to confirm the presence of substances known to cause inhibition in amplified assay systems. Target substances were leukocyte esterase, methemoglobin, lactoferrin, hydrogen peroxide, and lactic acid. Furthermore, immunoassays were preformed to detect the presence of the following inhibitors:

- Gamma interferon
- Mucosal IgA
- Non-target bacterial DNA

Data:

1) Comparison between True Positives using Reagent 1 and an untreated control

Number of collection sites: 4

- Collection site 1: Cervical Chlamydia (asymptomatic)
- Collection site 2: Urethral Gonorrhea (symptomatic)
- Collection site 3: Cervical Chlamydia(asymptomatic)
- Collection site 4: Urethral Gonorrhea (symptomatic)

Number of Samples that were Treated: 200 (50 from each collection site)

Number of Samples that were untreated: 200 (50 from each collection site)

Test Site #/ Assay	Number of Samples	Positives- **(Treated w/Reagent 1)**	Prevalence	Number of Samples	Positives- **Untreated control**	Prevalence
1 - LCx	50	8	16%	50	6	12%
2 - Probe-Tec	50	7	14%	50	4	8%
3 - TMA	50	5	10%	50	3	6%
4 - PCR	50	6	12%	50	3	6%
Totals :	200	**26**	**13%**	200	**16**	**8%**

2) GC/MS Cervical Data for Untreated Inhibited Specimens:

Lactoferrin >175g/mg

Methemoglobin >8mg/dl

Leukocyte esterase >15/μL

Lactic Acid: present, but not quantified

*All had statistically significant correlation with inhibited specimens

3) GC/MS Urethral data for Untreated Inhibited Specimens:

Neutrophil Esterase >15μl (achieved peaks)

Hydrogen peroxide: present, but not quantified

Zinc 110μg/dl

*All had statistically significant correlation with inhibited specimens

4) Immunoassay Data for Untreated Inhibited Specimens:

IgA cervical correlation

Gamma Interferon urethral and cervical correlation

Protein oxidation (hydroxy-nonenal) activity urethral correlation only

Results

1) Swabs impregnated with Reagent 1 yielded a statistically significant increase in amplification at all sites compared to a standard untreated swab.

2) There was no statistically significant difference between gonorrhea and chlamydia specimens with regard to their inhibition characteristics.

3) There was a statistically significant presence of target inhibitors in both untreated gonorrhea and chlamydia specimens.

4) Lactoferrin, hydrogen peroxide, methemoglobin, gamma interferon, lactic acid, leukocyte esterase were all associated with inhibited specimens.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of the present invention and are covered by the following claims. The contents of all references, issued patents, and published patent applications cited throughout this application are hereby incorporated by reference. The appropriate components, processes, and methods of those patents, applications and other documents may be selected for the present invention and embodiments thereof.